NITROGENOUS SUBSTANCE CHANGES IN THE NUTRITIVE BROTHS PREPARED FROM THE DRY RPD PREPARATION FOLLOWING CULTURING OF CL. BOTULINUM OF THE A, B, C AND E TYPES

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NITROGENOUS SUBSTANCE CHANGES IN THE NUTRITIVE BROTHS PREPARED FROM THE DRY KPD PREPARATION FOLLOWING CULTURING OF CL. BOTULINUM OF THE A, B, C AND E TYPES

[Following is the translation of an article by L. G. Ivanova and T. I. Sergeyeva, Gamaleya Institute of Epidemiology and Microbiology, AMN, USSR, Moscow, published in the Russian-language periodical Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii (Journal of Microbiology, Epidemiology and Immunobiology), Jan, 1964, pages 101-108. It was submitted on 12 Dec 1962. Translation performed by Sp/7 Charles T. Ostertag Jr.]

A study of the nutritional requirements of the botulism causative agent is extremely important for obtaining potent toxins and diagnosing the disease. One of the methodical approaches to the study of the nutritional requirements of the microbe is the investigation of changes taking place in the medium as a result of its growth.

At the present time, six types of botulism causative agent have been established -- A, B, C, D, E AND G. The nutritional requirements of each of these is complex and diverse. Clarification of the latter is very difficult during cultivation of the microbes on meat, gelatin, casein and other complex nutrient media. Nevertheless, on these media it was possible to show that for the growth of the botulism causative agent types A and B, tryptophan, leucine, isoleucine, valine, arginine, phenylalanine, tyrosine and methionine are required, and for representatives of type A tyrosine is essential and for type B -- histidine (Burrows, 1932; Fildes, 1935; Elberg, 1939; Mager et al, 1954 - 1956).

This data was only circumstantially confirmed by the study of the nutritional requirements of the botulism causative agent on synthetic (Burrows, 1933) and semisynthetic media (Inukai and Haga, 1960). The authors did not succeed in observing sufficiently good growth on these media, which testified to the inferiority of the latter. In addition to this, on these media it wasn't possible to detect toxin formation -- the most characteristic biological indicator for the botulism causative agent.

Thus, at the present time the problem concerning the role of nitrogenous substances in the process of growth and toxin formation of the botulism causative agent remains inadequately studied. Moreover, the above mentioned investigations were performed with bacilli of types A and B, and therefore the conclusions made on their basis cannot be used

in regards to other types of botulism causative agent.

Our mission included a comparative study of changes of nitrogenous substances in the medium caused by various types of Cl. botulinum.

In the experiments, broth was used which was prepared from a dry standard KPD preparation, the formula for which was published earlier (Ivanova et al, 1962). The stated preparation is a dry powder prepared from the hydrolysate of whale flour /K/, liver /P/, and yeast extract /D/. Broth made from this preparation is a rich nutrient medium on which the botulism causative agent of types A, B, C and E grow equally well and a sufficiently strong toxin is formed. This medium is different from other media used for the cultivation of anærobes by the great constancy of the chemical composition.

We established the studying of the nitrogenous substances in the medium according to the following chemical indicators: The amount of total, protein and amine nitrogen, peptone and tryptophane, and also by the qualitative change of the amino acid composition, determined in the medium by means of paper chromatography.

The dry preparation made from the hydrolysate of whale flour with liver and yeast extract was dissolved before use on the basis of 50g to 1 liter of distilled water. The broth obtained was checked for amine nitrogen content and pH. The amount of amine nitrogen in the medium was equal to 150 mg%, and the pH 8.0 - 7.8 before sterilization and 7.1 - 7.2 after sterilization. Then 70 ml of broth was poured into 100 ml sterile flasks with cotton and sterilized for 30 minutes at 110°. Before seeding, 0.5% glucose (a sterile 40% solution) was added.

Individual flasks with the medium were seeded with 0.5 ml of the following cultures of Cl. botulinum: Type A, Memphis strain and No. 98; type B, Nevin strain and No. 175; type C, strain No. 98 of two generations; and type E, strains No. 188-20. The inoculations of type A, B and C strains were incubated at 37°, and type E at 28° for five days. On the sixth day of cultivation the culture was centrifuged (20 min. at 3,000 rev./min.). The hyaline culturing liquid obtained was subjected to chemical and toxicological analysis.

The control in the tests was broth made out of the same series of dry preparations. It was prepared simultaneously with the test batch and placed in the incubator together with it.

In the control and test specimens, amine nitrogen was determined by the method of Zerensen--Gavrilov, peptone by the biuret reaction, tryptophan -- according to Peshkov (1943-1944), total and protein nitrogen --

according to the Kjeldahl technique; qualitatively the amino acid composition was established with the help of paper chromatography.

The liquid was desalted in order to determine the amino acids under investigation. For this, 10 ml of the liquid, containing around 30 mg% of amine nitrogen, was concentrated until dry and extracted with two portions (total volume -- 50 ml) of acetone, acidified with hydrochloric acid (1 ml of concentrated HCl to 100 ml of pure acetone). After removal of the acetone (evaporation into an exhaust hood) the dry residue was dissolved in 1 ml of distilled water and 0.3 ml of it was applied to a chromatogram. In the tests, Leningrad chromatographic (slow) paper was used. The solvent was a mixture containing butanol, acetic acid and water in the ratio of 4:1:5 (upper layer). Dispersal was performed in three steps at 8-10°. The developer was a 0.5% solution of ninhydrin in acetone with the addition of a 5% solution of acetic acid. The air dried chromatogram was maintained for 1-2 minutes at 80° up until complete development. The developed spots of amino acid were outlined with a black pencil and then the chromatogram was photographed.

The strength of the toxin was determined by titration on white mice following the intravenous introduction of 0.5 ml of diluted culturing liquid.

Eight series of the media were tested and the vigorous growth (with abundant gas formation) of all types of botulism causative agent was observed on them. The strength of the toxins in the culturing liquid for mice is presented in figure 1.

After incubation of the botulism causative agents, sharp changes were observed in the content of nitrogeneous substances in the medium -- tryptophan, peptone and amine nitrogen. After the incubation of all types of bacilli, the total and protein nitrogen remained without a change. The facts presented in table 2 include the results of 3-5 tests in one of the series of the strain being studied (No. 206). Analogous results were obtained in the remaining seven series.

Following incubation with type A and B botulism causative agents, the amine nitrogen in the medium increased by almost three times, and for type C -- by more than two times in comparison with the original content of nitrogen in the medium prior to seeding. The weakest increase of all was observed following the incubation of type E bacilli. The sharp increase of amine nitrogen in the culturing liquid after the activation of type E protoxin can be regarded more preferably as due to the action of pancreatin, however, this problem, just as the mechanism of activation, requires a special study.

The amount of peptone changed in reverse proportion to the amount of amine nitrogen established in the medium. The sharpest reduction in

the amount of peptone was noted following the incubation of types A and B bacilli, particularly the latter, and the least reduction following the incubation of type C bacilli; the causative agents of type E botulism, judging by the results obtained, did not consume peptone at all; somewhat of a reduction in it was noted only after activation which can be described as the result of the destructive action of pancreatin.

Most significant were the data obtained when determining the amount of tryptophan in the medium. After the sixth day of growth of representatives of types A and B in the culturing liquid, tryptophan was not detected, after incubation of type C its content was reduced by approximately two times, and type E bacilli consumed tryptophan in an insignificant amount. After activation of type E protoxin, the content of tryptophan in the culturing liquid exceeded its quantity in the initial medium.

It was established that the whale-liver medium with yeasts contained 16-19 amino acids which are determined chromatographically (figure 1): Lysine (2), arginine (4), aspartic (5) and glutamic (9) acid, glycocoll (7), threonine (10), α-, β-alanine (11), tyrosine (13), methionine (16), valine (17), isoleucine (19), leucine (20). In small quantities it was possible to detect serine (6) and arginine (4).

With a comparative study of the amino acid composition of broth prepared from the dry whale-liver hydrolysate, and the same broth after incubation in it of botulism causative agents, types A, B, C and E, significant differences appeared (figures 2 and 3). The most consistent changes were observed in the amino acid composition of the broth after incubation of types A and B bacilli. The amount of glycocoll, aspartic acid, tyrosine, leucine, glutamic acid, threonine, and α-, β-analine increased (particularly noticeable in the last three). The amount of lysine, histidine and arginine decreases somewhat. Phenylalanine appeared during the incubation of type A representatives on the medium and sometimes with type B.

During incubation of type C botulism causative agents the amount of glutamic and aspartic acid as well as glycocoll and threonine remained unchanged. The amount of leucine, isoleucine, methionine, valine, and α-, β-analine increased, just as during incubation of types A and B. The amount of glutamic acid, threonine, aspartic acid and glycocoll increased less sharply than during incubation of types A and B bacilli. The amount of lysine, histidine and serine was reduced to traces and arginine disappeared completely. Phenylalanine did not appear during incubation of type C bacilli on the medium, in contrast to type A and sometimes type B (see figure 3).

After the growth of type E representatives, results were noted which were analogous to those observed during incubation of types A and

B, but phenylalanine never appeared, as this was noted in the case of incubation of type C. In contrast to all the others, type E bacilli promoted the appearance of hydroxyproline in the medium. However, such amino acids as glutamic acid, threonine, α-, β-alanine, methionine and valine were detected after activation of the culturing liquid obtained from the incubation of type E botulism causative agents in the same quantities as before activation; after activation hydroxyproline disappeared.

The pH was determined at the same time as the content of nitrogenous substances in the medium. After 24 hours from the beginning of cultivation, when vigorous growth and profuse gas formation were already noted, the medium had a more acidic reaction -- the pH fluctuated from 5.9 to 6.2 for the various types of botulism causative agent. The strength of the toxin at this time did not exceed 500-1000 Dlm in 1 ml. On the sixth day the medium was over-alkaline and the activity of the toxin reached the maximum value. The pH of the medium acquired the value of the initial index and during incubation of representatives of types A and B even exceeded it.

Proteolytic microorganisms, such as Cl. botulinum, grow well and form toxins on media with a shallow splitting up of protein, the hydrolysate of which is used for preparation of the medium (Kozlov, 1950). In literature there are indications (Kindler et al., 1956) of the necessity of protein substances for the growth of botulism causative agents. The data obtained by us serves as an indirect support of this. Under the effect of proteolytic enzymes the botulism causative agents broke down peptone and other products of protein destruction. As a result of this there was a vigorous increase in the amount of amine nitrogen in the medium. This was observed during chromatographic analysis of the amino acid composition of the medium. Gaseous ammonia was not formed apparently, since the amount of total nitrogen in the medium after incubation did not change.

In a comparison of the nature of change in the nitrogen composition of the medium under the influence of growth and the toxin formation of various types of botulism causative agent, it was established that types A and B most actively consume the peptone and tryptophane of the medium. During this a sharp increase of amine nitrogen was observed. Proteolytic activity in type E was weakly expressed: The extent of amine nitrogen in the medium following its cultivation was the lowest, peptone was detected in the medium in the same quantity as prior to incubation, and the content of tryptophane changed insignificantly. In this respect, type C occupied an intermediate position between types A--B and E.

The data, obtained as a result of studying changes of the amino acid composition of a whale-liver medium with yeast after the growth of botulism causative agents type A, B, C and E on chromatograms, showed that

many amino acids apparently take an active part in the processes of growth and toxin formation. Thus the amount of such amino acids as glycocoll, threonine, glutamic and aspartic acid increased sharply after the incubation of type A, B and E bacilli, remaining unchanged following the incubation of type C botulism causative agent.

Note must be made of the decrease and disappearance from the medium of several amino acids -- serine, arginine, lysine, histidine, etc. Apparently these amino acids may have considerable importance in the process of growth and toxin formation. This is confirmed by published data (Burrows, 1932-1933; Mager, Kindler, Grossowicz, 1954-1956; and others). A number of amino acids appear in the medium only after incubation of specific types of botulism causative agent: Phenylalanine following the incubation of type A, sometimes B, and hydroxyproline following the incubation of type E.

However, the data obtained is insufficient for determining the role of the stated nitrogenous compounds in the process of growth and toxin formation of botulism causative agents. This can be cleared up with a more detailed study.

Conclusions

1. During a study of changes of nitrogenous substances in a dry whale-liver medium with yeasts, their active participation was exposed in the processes of growth and toxin formation of type A, B, C and E botulism causative agents.

2. The amount of nitrogenous substances determined in the medium such as amine nitrogen, peptone, tryptophane and other amino acids, following incubation and the formation of botulin toxin of types A, B, C and E is a characteristic for each type. Thus the amount of amine nitrogen during cultivation of microbes of types A and B increased by three times, type C -- two times, and type E -- one and one half times in comparison with the original medium; the amount of peptone in the medium decreased most significantly during the growth of types A and B, less during the incubation of type C, and hardly changed following the cultivation of type E; tryptophane completely disappeared in the medium following the growth of type A and B bacilli, half the amount remained following the incubation of type C, and it was consumed insignificantly by type E.

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[The following English summary appears with the Russian article.]

By using the standard medium prepared from the dry KPD preparation a study was made of the changes of chemical indices of nitrogenous substances in this medium resulting from the growth of and toxin formation by Cl. botulinum of the A, B, C and E types. As demonstrated, the quantity of nitrogenous substances of the medium (amine nitrogen, peptone, tryptophane and of some other amino acids) changes characteristically for each of the Cl. botulinum types and in accordance with the extent of its proteolytic intensity. Amine nitrogen content increases in growing Cl. botulinum of the A and B types 3-fold, of the C types -- 2-fold, and of the E type - 1½ times. The peptone level in the medium also goes down inversely to the amine nitrogen content. Tryptophane completely disappears while culturing A and B types, is halved in the growing of the C type and remains almost unchanged with the type E.

Table 1

Strength of toxins in a cultured liquid obtained after incubation of different types of botulism causative agents

Type	Strain	Strength of toxin (Dlm in 1 ml)
A	Memphis	20,000 -- 50,000
	No 98	100,000
B	Nevin	20,000 -- 50,000
	No 175	100,000
C	No 91, first generation	50,000 -- 100,000
	No 91, second generation	100,000
E	No 188-20, before inactivation	100
	No 188-20, after inactivation with pancreatin	10,000

Table 2

Changes in nitrogenous substance content in a medium during cultivation of botulism causative agents

Type	Strain	Amount (mg%) in medium				
		Amine nitrogen	Peptone	Tryptophan	Common nitrogen	Protein nitrogen
A	Memphis	473	2 225	None	980	168
	No 98	300	2 400	None	980	168
B	Nevin	446	1 900	None	980	168
	No 175	450	2 000	None	980	168
C	No 91	354	2 300	38	980	154
	First and second generation					
E	No 188-20 before activation	306	2 900	72	980	168
	Control of medium (series No 206 before seeding)	161	3 000	92	980	168

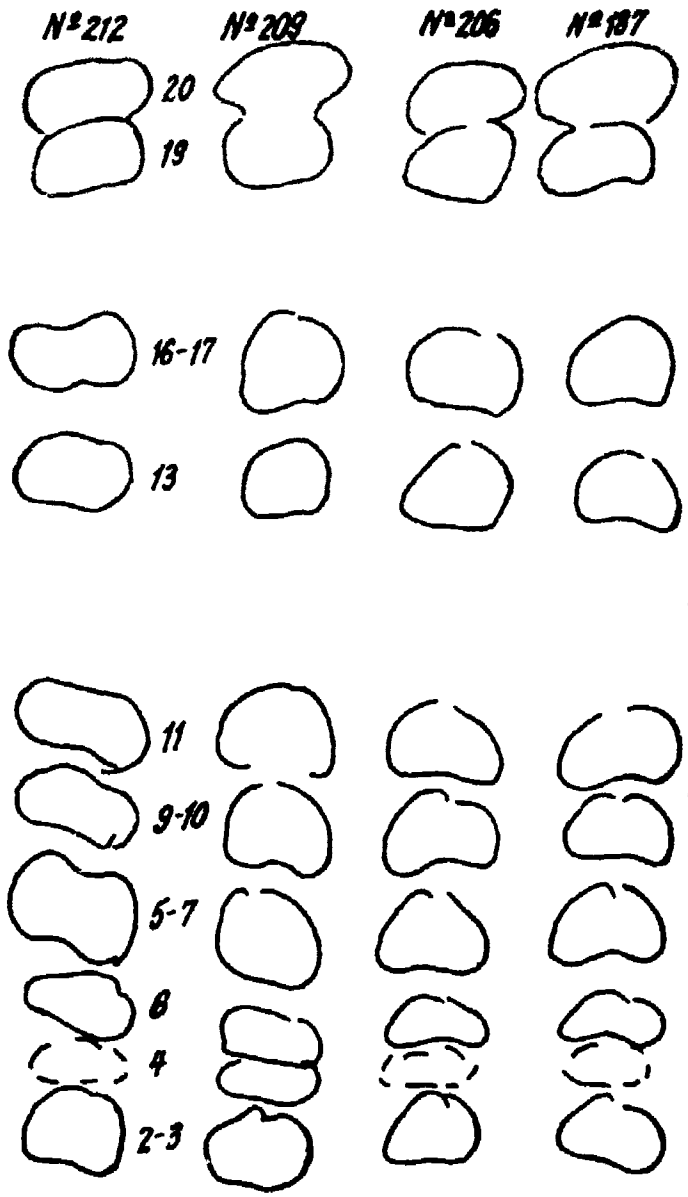

Figure 1. Chromatogram of the amino acid composition of a medium of whale-liver hydrolysate with yeast extract (series No 187, 206, 209, 212).

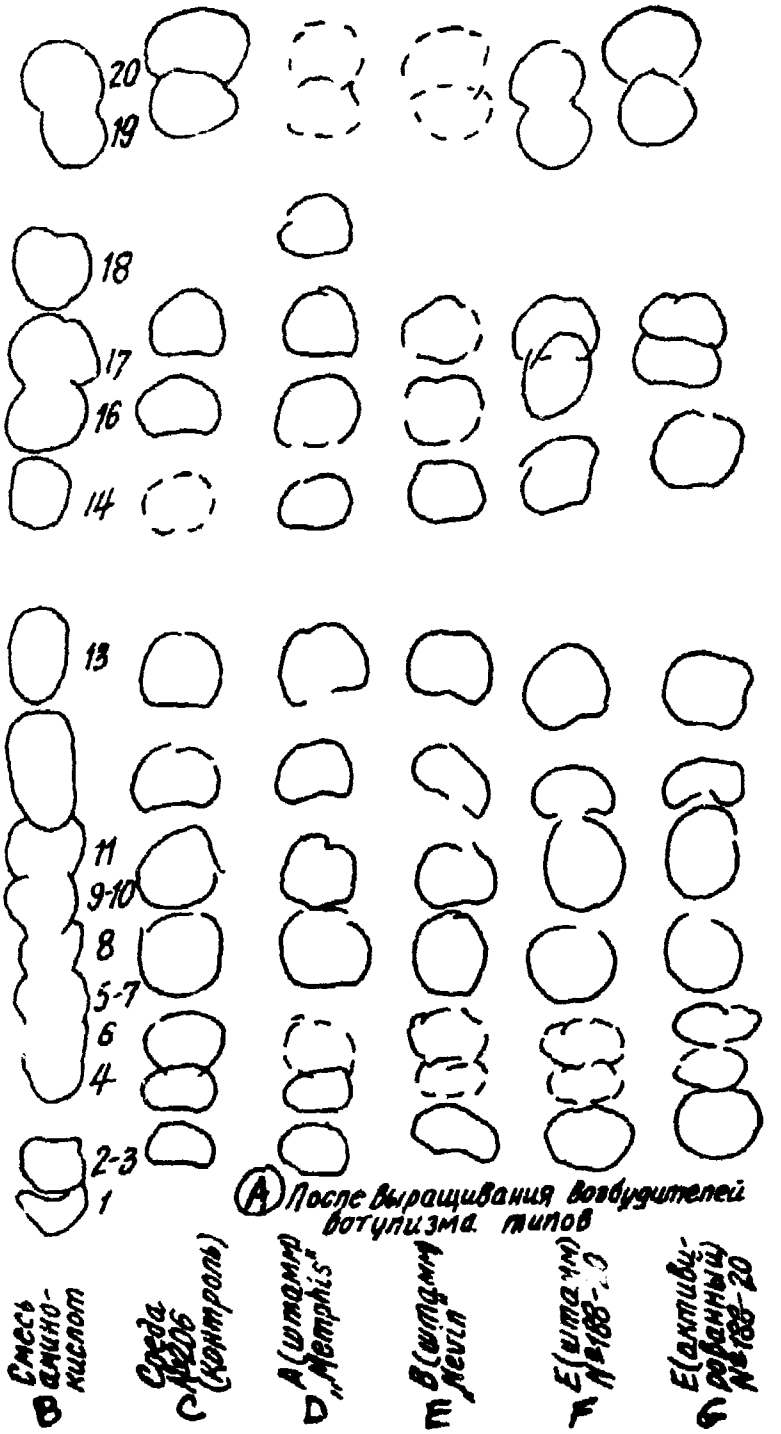

Figure 2. Changes in the amino acid composition of a medium (series No 212) after incubation with the botulism causative agents, types A, B, C and E. (A) -- After incubation with different types of botulism causative agent; (B) - Mixtures of amino acids; (C) - Medium No 206 (Control); (D) - A (strain) "Memphis"; (E) - B (strain) "Nevin"; (F) - E (strain) No 188-20; (G) - E (activated) No 188-20.

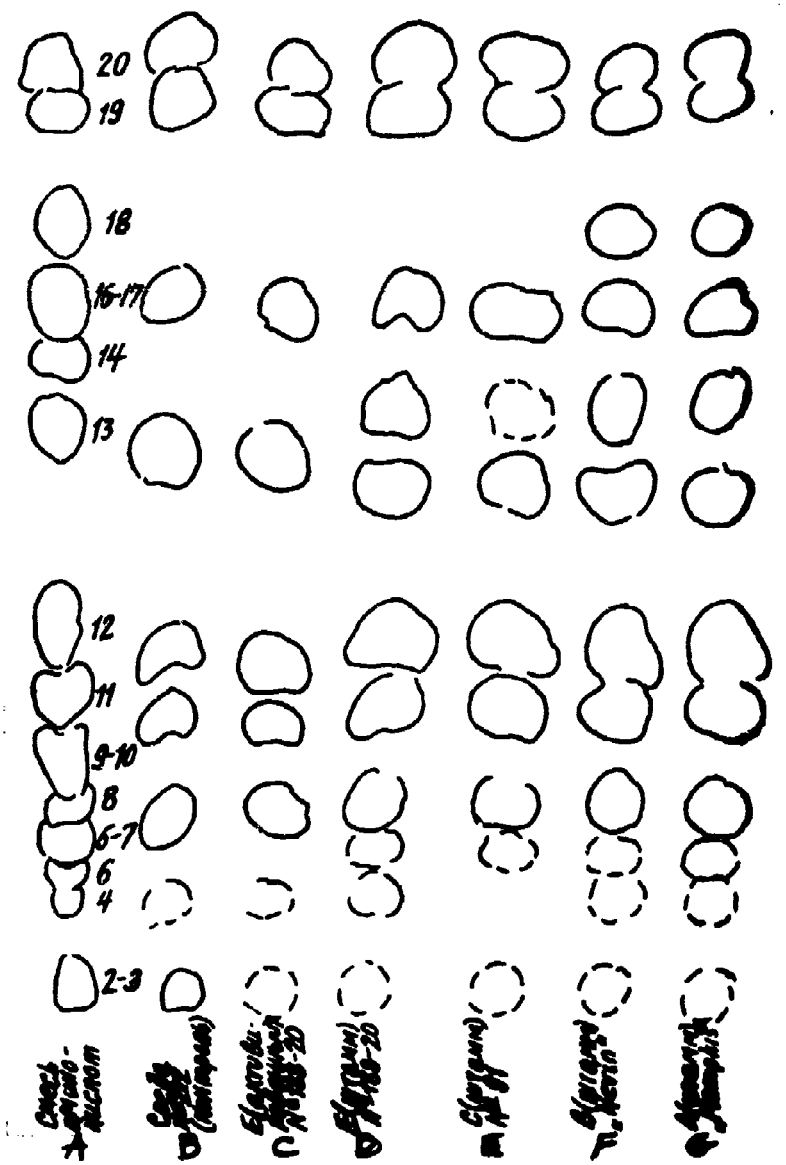

Figure 3. Changes in the amino acid composition of a medium (series No 206) after incubation with the botulism causative agents, types A, B and E. (A) - Mixture of amino acids; (B) - Medium No 212 (Control); (C) - E (activated) No 188-20; (D) - E (strain) No 188-20; (E) - C (strain) No 91; (F) - B (strain) "Nevin"; (G) - A (strain) "Memphis".